Title: Cloning the gate and RFP onto pSB1C3

Goal: To insert the RFP RBS and gene behind the logic gate.
Date range: 7/23 - 7/27
Members that worked on project: Josephina Hendrix

Summary: The logic gate was of our first design: a strong constitutive promoter upstream of a terminator that was flanked by the integrase recognition sites. We successfully ligated this gate and an RBS-RFP onto a pSB1C3 backbone. The results were gel and sequence confirmed.

New part number: This part was not submitted

Starting status of DNA: the logic gate was previously inserted into pSB1C3 by Daren.
PCR done by Naomi:

- Purpose: To remove the promoter and terminators from BBa_J04450
- PCR polymerase: Q5
- PCR of: BBa_J04450

Reagent	**50 ul reaction**	**Concentration**
5x Q5	10 ul	1 x
10 mM dNTPs	1 ul	200 uM
10 uM F. Primer	2.5 ul	0.5 uM
10 uM R. Primer	2.5 ul	0.5 uM
DNA (concentration)	1ul	0.5ng
Q5 polymerase	0.5 ul	0.02 U/ul
Water	22.5 ul	

Reaction conditions:

Step	**Temp (C)**	**Time**
I. Denature	98	5 min.
35 cycles	98	10 s
	64	30 s
	72	30 s
F. Extension	72	2 min.

- Primers used: GEM027 F&R

PCR clean-up:

- Elution volume: 30ul
- Concentration: 34.8 ng/ul

Digestion:

- State purpose: To cut and get extract the gate and RFP for subsequent ligation.
- Digestion of: our logic gate on pSB1C3

Reagent	**50 ul reaction**	**Concentration**
Cutsmart Buffer	5 ul	1 x
SpeI-HF	1 ul	10 units
PstI-HF	1 ul	10 units
DNA (78.3 ng/ul)	6.4 ul	500 ng
Water	36.6 ul	

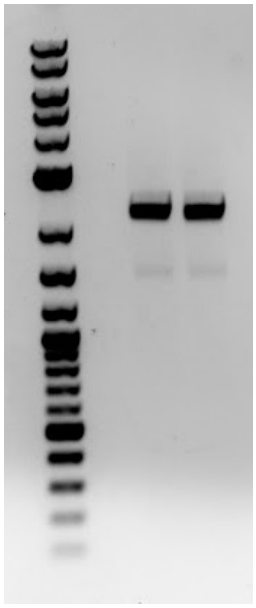
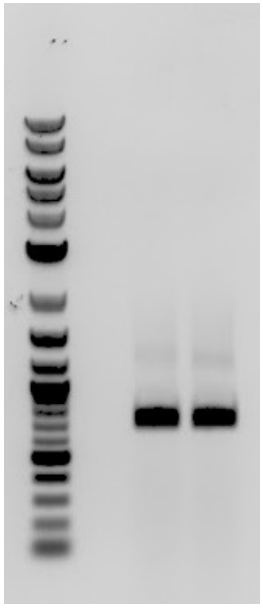
- Digestion of: PCR product (BBa_J04450 without its promoter, terminator, or vector)

Reagent	**25 ul reaction**	**Concentration**
Cutsmart Buffer	2.5 ul	1 x
XbaI	.5 ul	10 units
PstI-HF	.5 ul	10 units
DNA (34.8 ng/ul)	6.5 ul	~226 ng
Water	15 ul	

- Reaction condition: Reactions were incubated at 37C for 1 hour then heat inactivated at 80C for 20 minutes

Gel:

- Latter: 2-log
- Gel concentration: .8%
- Expected sizes:
    - Gate and pSB1C3 should be ~2500bp
    - RFP part should be about ~750bp

- Gel of pSB1C3 backbone:
- 
- Gel of RFP part
- 

Gel extraction:

- Extracted: the gate-pSB1C3 and RBS-RFP
- Followed the Qiagen gel extraction protocol; no problems

Ligation:

- Used the digestions of our logic gate on pSB1C3

Reagent	Amount (20ul)
T4 Ligation Buffer	2 ul
gate-pSB1C3 (gel-extracted)	2.5 ul
RBS-RFP (gel-extracted)	7 ul
Water	7.5 ul
T4 ligase	1 ul

- Incubated for 14 hours at 16C then heat inactivated for 10 minutes at 65C

Transformation:

- Used the commercial cells from NEB. 8ul of the ligation product was transformed into 40ul of these cells. As a negative control, 10 uls were run through the protocol but no DNA was added.
- Cells were heat shocked at 42C for 30s then recovered for 110 minutes.
- Cells were then plated on LB+Chlor (170): 100ul of the cells transformed with DNA and 200ul for the negative control

Overnight:

- Results of transformation: No growth for negative control plate. ~40 colonies grew for the sample.
- 4 colonies were chosen for overnights. The overnights were composed of 6mL of LB+Chlor (170)

Mini-prep:

- There were no incidences with the mini-prep

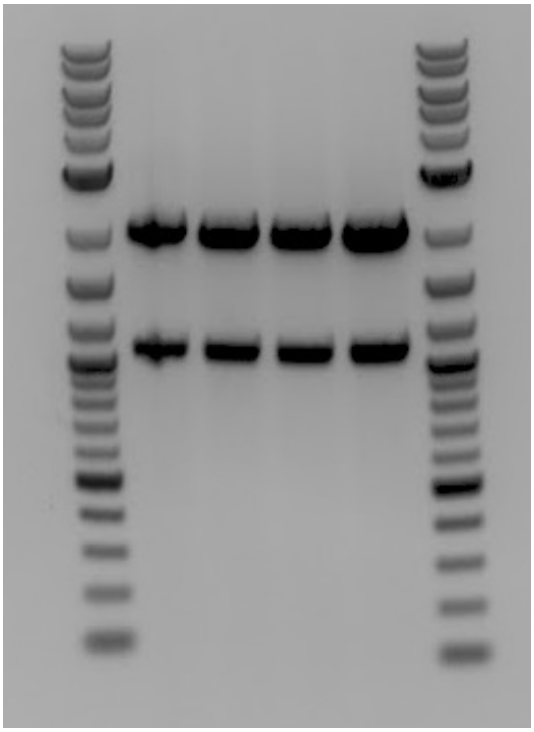
Digestion:

- Purpose: To check the plasmid sizes from the overnight
- Digestion of: gate-RFP-pSB1C3

Reagent	10 ul reaction	Concentration
Cutsmart Buffer	1 ul	1 x
EcoRI-HF	0.2 ul	2 units
PstI-HF	0.2 ul	2 units
DNA (concentration)	1 ul	100 ng
Water	7.6 ul	

Gel:

- Expected sizes:
    - pSB1C3: 2070 bp
    - gate-RFP: ~1050 bp



Based on the gel, we do have the desired part.

Upon further investigation we found that the cells, without integrase, did turn slightly red. This indicates that the terminator on the gate is leaky. The leakiness was also seen with the GFP. In order to remedy this problem, we will try transferring this part onto a lower copy plasmid and try a second operator where the promoter is between the Bxb1 recognition sites.

Sequencing:
The gate and RFP were previously sequenced individually and were correct. This new part was not sequenced due to its leakiness so we do not know if the gate was somehow flipped.

Part submission:

- This part was not submitted.